

The target binding region may have any convenient number of additional bases at its 5' end. All or some of these additional bases may form part of any region(s) of internal hybridisation.

In a further aspect of the invention the primer may comprise a capture region. This may be placed at any convenient location, preferably on the primer tail. The capture region may be a contiguous or branched structure (cf. Figure 8c) The capture region hybridises to complementary sequence on, for example a solid phase.

Any convenient template dependent polymerase may be used, this is preferably a thermostable polymerase enzyme such as taq, more preferably taq Gold.

Similarly any convenient nucleoside triphosphates for conventional base pairing may be used. If required these may be modified for fluorescence. As these may affect polymerisation rates up to only about 1 in 20 dNTPs used is modified for best results.

Further details of convenient polymerases, nucleoside triphosphates, other PCR reagents, primer design, instruments and consumables are given in "PCR" by C.R. Newton and A. Graham (The Introduction to Biotechniques series, Second Edition 1997, ISBN 1 85996 011 1, Bios Scientific Publishers Limited, Oxford). Further guidance may be found in "Laboratory protocols for mutation detection" edited by Ulf Landegren, published by the Oxford University Press, Oxford, 1996, ISBN 0 19 857795 8.

The invention will now be further illustrated by the following non-limiting specific description wherein the tailed primers of the invention are referred to as *Scorpions* primers:

The design of *Scorpions* primers may follow well known guidelines for PCR amplimers; the 3' end of the *Scorpions* primer and/or the target binding region may taken directly from, for example an existing PCR or ARMS assay.

Target binding regions are typically about 17 bases (DNA) although (depending upon the temperature at which measurements are to be taken) shorter (as little as 6 to 10 bases) target binding regions be used. In this context we envisage that non-natural nucleic acids such as PNA or 2'-O-alkyl-RNA (particularly 2'-O-methyl RNA) will be useful since they have higher T_m s when bound to their targets. The spacing on a DNA strand between the amplicon binding region and its complementary sequence within the amplicon may be as little as 30 bases (that is directly abutting the primer region) or may be as much as about 200-300 bases. The efficiency of the unimolecular interaction is expected to decline as this distance increases.

Where stem regions are used, they may range from 2 bases (especially useful for 2'-O-methyl RNA or PNA) to about 6, 8, 10 or more bases. The balance between stem length and amplicon binding length is important: the probe-target complex should have a stronger (more negative) ΔG (free energy) than the stem duplex at the assay temperature.

5 Any polymerisation blocking group such as those described in our EP-B1-0 416817 (Zeneca Limited) is suitable. However, we prefer that it should be easily incorporated by solid phase oligonucleotide chemistry and should also form a substrate for further extension in the same chemistry. Convenient examples include hexethylene glycol (HEG) and tetraethylene glycol (TEG) phosphoramidites.

10 The range of assays which can be performed using the *Scorpions* primers is extensive. Detection may, for example, be effected after PCR amplification and at room temperatures since the unimolecular hybridisation events happen quickly and are stable for extended periods (at least overnight). Furthermore, positive fluorescence signals are so high and backgrounds so low, that fluorescence can be observed by eye under appropriate illumination and at ambient temperature. These are significant advantages.

Where allelic discrimination is employed as an endpoint, this may require the use of temperature control to selectively destabilise mismatched target.

15 The *Scorpions* primers of this invention are particularly suited to real time assays since signal generation is rapid and requires only a unimolecular interaction. Additionally, backgrounds are low and signals are high allowing a good deal of flexibility in assay design. Continuous monitoring of fluorescence through the PCR is possible with the appropriate hardware.

20 *Scorpions* primers also have substantial benefits for *in situ* techniques such as *in situ* PCR (ISPCR) and primed *in situ* synthesis (PRINS). Only priming events which generate the desired product produce signal and this provides substantial benefits over other techniques for detecting products within a cell.

25 It is generally desirable to include the *Scorpions* primer at the beginning of the reaction and to measure fluorescence in the closed tube (homogeneous) either continuously or post-PCR. Alternatively the *Scorpions* primer may be added at a later stage of the amplification; the only requirement is that the *Scorpions* primer must undergo a single round of extension and produce the unimolecular tail/target duplex.

Using appropriate signalling systems (for example different fluorophores) it is possible to combine (multiplex) the output of several *Scorpions* primers in a single reaction. The number of primers that may be used is limited only by experimental considerations.

We now disclose the following non-limiting embodiments:

5 **Fluorophore/Quencher embodiment**

See Figure 1. Quenching is achieved by the random folding of the tail bringing the fluorophore/quencher (F/Q) pair into proximity by chance (Figure 2). In order to maximise this quenching, it is preferred but not essential to have the fluorophore in the middle of the molecule with the quencher at the 5' end. Signal "switch-on" is by the same loss of quenching caused by hybridisation of the probe (Figure 3). In this embodiment, it is not critical that the F and the Q are at opposite ends of the probing entity, and it may be beneficial to place them closer together within the probe portion. It is important, though, not to disrupt the target binding function of the tail by introducing bulky, non base-pairing elements, but both fluorophore and quencher could be introduced on uracil monomers, replacing thymidines in the probe. We believe that this embodiment may work best as an amplicon detector.

10 **Intercalation embodiment**

In this embodiment, the design of the *Scorpions* primer is further simplified, having no quencher involved. Instead, the tail carries an intercalating dye which is capable of being incorporated between the bases of a double stranded nucleic acid molecule, upon which it becomes highly fluorescent. In this way, sequence specific intercalation is achieved (Figure 4a,b). In contrast to the "no-stem" method described in the previous embodiment, the intercalating fluorophore is better placed at the 5'-terminus of the *Scorpions* molecule or as an internal part of the loop. Internal folding within the primer is best minimised to ensure the absence of double stranded DNA which may then be intercalated, leading to high background noise. If the dye is placed within the loop portion of the molecule, it may be possible to have a hairpin structure (which would enhance the allele specificity of the the hybridisation). The dye used is preferably not a standard fluorophores but rather an intercalator having low fluorescence in the absence of double stranded target and a high enhancement when intercalated. Suitable fluorophores include the cyanine dyes developed by Molecular Probes, ethidium bromide, acridine and others. The dyes may need to be modified to ensure their easy

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